K:\EMATA\BIDMC\98-20\APPLICAT
WP 70
PG/EWM/jaw/dc
December 9, 1998

PATENT APPLICATION
Docket No.: BIDMC98-20

-1-

Date: 12/9/98   Express Mail Label No. EL015516025US

| Inventors: | Jan E. Schnitzer and Philip Oh |
| Attorney's Docket No.: | BIDMC98-20 |

IMMUNOISOLATION OF CAVEOLAE

GOVERNMENT SUPPORT

BACKGROUND OF THE INVENTION

Cholesterol and glycolipids self-associate in lipid bilayers to form organized compositional microdomains (Thompson, T.E., *et al., Annu. Rev. Biophys. Chem. 14*:361 (1985)). Glycosyl-phosphatidylinositol (GPI)-anchored proteins and other lipid-

10   linked proteins may preferentially partition into glycolipid microdomains that are resistant to nonionic detergent solubilization (Schroeder, R., *et al., Proc. Natl. Acad. Sci. USA 91*:12130 (1994); Brown, D.A. and Rose, J.K., *Cell 68*:533 (1992); Letarte-Murhead, M. *et al., Biochem. J. 143*:51 (1974); Hoessli, D. and Runger-Brandle, E., *Exp. Cell. Res. 166*:239 (1985); Hooper, N.M. and Turner, A.J., *Biochem. J. 250*:865

15   (1968); Sargiacomo, M. *et al., J. Cell. Biol. 122*:789 (1993); Lisanti, M.P. *et al., J. Cell. Biol. 123*:595 (1993)). GPI-anchored proteins appear to be sorted into glycolipid, detergent-resistant "rafts" in the trans-Golgi network for polarized delivery to the cell surface by smooth exocytotic carrier vesicles which are resistant to detergents and also contain caveolin (Brown, D.A. and Rose, J.K., *Cell 68*:533 (1992); Sargiacomo, M. *et

20   al., J. Cell. Biol. 122*:789 (1993); Lisanti, M.P. *et al., J. Cell. Biol. 123*:595 (1993);

Brown, D. *et al., Science 245*:1499 (1989); Simons, K. and van Meer, G., *Biochemistry 27*:6197 (1988); Garcia, M. *et al., J. Cell Sci 104*:1281 (1993); Kurzchalia, T.V. *et al., J. Cell Biol. 118*:1003 (1992); Dupree, P. *et al., EMBO J. 12*:1597 (1993); Hannan, L.A. *et al., J. Cell. Biol. 120*:353 (1993)). Caveolae are smooth membrane invaginations that

5   are also resistant to detergent extraction; they exist on the surface of many different cell types, and are especially abundant in endothelium. Caveolae are apparently also rich in glycolipids, cholesterol, and caveolin (Kurzchalia, T.V. *et al., J. Cell Biol. 118*:1003 (1992); Schnitzer, J.E. *et al., Proc. Natl. Acad. Sci. USA 92*:1759 (1995)). Low-density, Triton-insoluble membranes are frequently equated with caveolae (Sargiacomo, M., *et*

10 *al., J. Cell Biol. 122*:789 (1993); Lisanti, M.P. *et al., J. Cell. Biol. 123*:595 (1993); Chang, W.-J. *et al., J. Cell. Biol. 126:*127 (1994); Lisanti, M.P. *et al., J. Cell. Biol. 126*:111 (1994)), but recent work has shown they have a mixture of detergent-resistant microdomains (Schnitzer, J.E. *et al., Science 269*:1435-1439 (1995)). Characterization of caveolae shows that they are very enriched in caveolin; the glycolipid GM1; the

15 plasmalemmal CA2+-dependent adenosine triphosphatase; and the inositol 1,4,5-triphosphate receptor; these four molecules have all been shown by independent means to reside on the cell surface almost exclusively in caveolae (Dupree, P., *et al., EMBO J. 12*:1597 (1993); Parton, R.G., *J. Histochem. Cytochem. 42*:155 (1994); Rothberg, K.G. *et al., Cell 68*:673 (1992); Montessano, R . *et al., Nature 296*:651 (1982); Fujimoto, T.,

20 *J. Cell.Biol. 120*:1147 91993)) and thus represent key markers of caveolae.

Caveolae have been implicated not only in signaling but also in transport via endocytosis, transcytosis, and potocytosis (Montessano, R. *et al., Nature 296*:651 (1982); Schnitzer, J.E., *Trends Cardiovasc. Med. 3*:124 (1993); Oh, P. *et al., J. Cell Biol. 127*:1217 (1994); Schnitzer, J.E. and Oh, P., *J. Biol. Chem. 269*:6072 (1994);

25 Schnitzer, J.E. *et al., Proc. Natl . Acad. Sci. USA 92*:1759-1763 (1995); Schnitzer, J.E. *et al., J. Biol. Chem. 270*:14399-14404 (1995); Millci, A.J. *et al., J. Cell Biol. 105*:2604 (1987); Anderson, R.G. W. *et al., Science 265*:410 (1992)). However, there is disagreement as to whether caveolae serve as signaling centers (see Liu, J. *et al., J. Biol. Chem 272*:7211-7222 (1997), Schnitzer, J.E. *et al., Mol. Biol. Cell 5*:75a (1994);

Schnitzer, J.E. *et al.*, *Proc. Natl . Acad. Sci. USA 92*:1759-1763 (1995); Schnitzer, J.E. *et al.*, *J. Biol. Chem. 270*:14399-14404 (1995); contrast with Stan, R.-V. *et al.*, *Mol. Biol. Cell 8*:595-605 (1997)). The exact physiological composition and functions of caveolae remain undefined.

5   SUMMARY OF THE INVENTION

The present invention is drawn to methods of producing purified caveolae, as well as the purified caveolae produced by the methods, and uses of the purified caveolae. In the methods, immunoisolation of caveolae is performed, using an antibody that is specific for caveolin and that is able to bind oligomerized caveolin found around

10   intact caveolae. Immunoisolation can be performed on a wide variety of starting materials, including cells of interest, such as cultured cells or cells isolated from a tissue; a tissue itself; cell lysate; microsomes derived from cells or from tissue; or a sample of plasma membranes.

In one embodiment, the starting material can be subjected to membrane

15   disruption method and/or a preliminary separation step prior to the immunoisolation. If such a separation step is performed, the separation is based on a physical characteristic of cell membranes (for example, density, size, or phase separation), in order to provide a starting material containing a concentrated amount of plasma membranes. The initial separated fractions (e.g., the lowest density fractions in a density separation) are then

20   collected, and subjected to the immunoisolation method to separate caveolae from other materials in the initial fractions.

During the immunoisolation, a sample of interest (e.g., the starting material, sample of plasma membranes, or initial fractions) that comprises plasma membranes is incubated, preferably for a brief time period (e.g., for less than approximately 2 hours,

25   preferably for approximately one hour or less), with the antibody that is specific for caveolin. Caveolae that are bound to the antibody are then separated from other materials in the sample of interest, thereby producing purified caveolae.

In a preferred embodiment, a sample of plasma membranes from cells of interest is used as the sample of interest. The sample of plasma membranes can be subjected to a membrane disruption method, such as sonication or shearing, to produce disrupted plasma membranes. If desired, the disrupted plasma membranes can then be subjected

5   to separation based on a physical characteristic of the membrane, as described above. The resultant material is then subjected to the immunoisolation method to separate caveolae from other materials in the initial fractions.

The methods of the invention provide simplified, efficient means to produce purified caveolae, while minimizing contamination and avoiding loss of molecules that

10   dissociated from caveolae with time. The caveolae produced by the methods more closely resemble caveolae in their native state; as the methods eliminate extended immunoisolation methods which would otherwise result in significant loss of various caveolar components as well as adsorption of nonspecific proteins or contaminating membranes. The methods can be used to produce purified caveolae from a wide variety

15   of cells or tissues, including not only endothelial cells and tissues, but also other (non-endothelial) cells and tissues, as well as cultured cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphic representation of the binding equilibrium over time of monoclonal antibody CAV to caveolin as it exists around caveolae in plasma

20   membranes (i.e., in oligomerized form).

Figure 2 is a graphic representation of the percent of binding of antibodies CAV, 2297 and Z034, as well as polyclonal antiserum pAB, to caveolin as it exists around caveolae in plasma membranes (i.e., in oligomerized form).

DETAILED DESCRIPTION OF THE INVENTION

25   The present invention relates to purified caveolae, methods of producing the purified caveolae; and uses for the purified caveolae. As described herein, various techniques to isolate caveolae were compared to determine the effects of the techniques

on the molecular composition of the final isolated caveolae. It was discovered that a brief immuno-affinity isolation procedure, using an antibody that was specific for caveolin and which bound with high affinity to caveolin in its native state as an oligomeric structure, allowed a simplified and quick isolation of caveolae. Caveolae

5   isolated by this procedure retained more caveolae-related proteins than those isolated by other procedures, and thus were more representative of caveolae in their native state than caveolae isolated by other procedures.

As a result of this discovery, methods are now available to quickly and efficiently isolate caveolae. The methods can be carried out on any tissue, cell type, or

10   cellular fraction whose plasma membranes contain the desired component (caveolae). For example, endothelial cells from representative tissues such as vascular, pulmonary, cardiac, cerebral, nephric, hepatic and endocrinous tissue, including the vascular system, lung, heart, liver, kidney, brain, and other organs, can be used. Alternatively, cells from any other type of tissue can be used, including non-endothelial cells. Cultured cells,

15   cells isolated from a tissue, or a cell lysate can also be used, as can a tissue itself, as well as microsomes derived from cells or from a tissue. The tissue, cells, cell lysate, or microsomes from which the caveolae are isolated is referred to herein as the "starting material".

In the methods of the invention, a sample of interest (e.g., "starting material")

20   that comprises plasma membranes is used. If desired, plasma membranes can first be separated from the other components of the tissue, cells, cell lysate, or microsomes used as the starting material, before immunoisolation is performed. A "sample of plasma membranes," as used herein, refers to plasma membranes that have been separated from other components of the starting material. The sample of plasma membranes can

25   obtained from the starting material by any appropriate method, such as silica coating methods (see U.S. patent 5,281,700; Schnitzer, J.E. *et al., Proc. Natl. Acad. Sci. USA* 92:1759-1763 (1995); Schnitzer, J.E. *et al., J. Biol. Chem.* 270:14399-14404 (1995); Schnitzer, J.E. *et al., Science* 269:1435-1439 (1995); Schnitzer, J.E. *et al., Mol. Biol. Cell* 5:320(a) (1994); or Schnitzer, J.E. *et al., Mol. Biol. Cell* 5:75a (1994)), or density

gradient centrifugation methods (see, e.g., Smart, E.J. *et al., Proc. Natl. Acad. Sci. USA 92*:10104-10108 (1995); *Biological Membranes: A Practical Approach* (Findlay and Evans, Eds., IRL Press at Oxford University Press, Oxford, England, 1987); *Subcellular Fractionation: A Practical Approach* (Graham and Rickwood, Eds., IRL Press at

5   Oxford University Press, Oxford, England, 1997); *Cell Biology: A Laboratory Handbook* (J. Celis, Ed., 2nd Edition, Acad. Press, Inc., San Diego, CA, USA, 1998), especially "Isolation and Subfractionation of Plasma Membranes to Purify Caveolae Separately from Glycol-phosphatidylinositol-anchored Protein Microdomains" (P. Oh and J.E. Schnitzer, pp. 34-45)). Alternatively, methods that utilize physiological release

10   of caveolae by inducing budding from plasma membranes can be used to obtain the sample of plasma membranes (see, e.g., Schnitzer, J.E. *et al., Science 274*:239-242 (printer's erratum, *Science 274*:1069) (1996)).

If desired, the starting material comprising plasma membranes, or the sample of plasma membranes, can be subjected to a membrane disruption method, to produce a

15   disrupted plasma membrane sample. The membrane disruption method can be sonication, shearing, or another appropriate membrane disruption method (see, e.g., *Biological Membranes: A Practical Approach* (Findlay and Evans, Eds., IRL Press at Oxford University Press, Oxford, England, 1987); *Subcellular Fractionation: A Practical Approach* (Graham and Rickwood, Eds., IRL Press at Oxford University

20   Press, Oxford, England, 1997); *Cell Biology: A Laboratory Handbook* (J. Celis, Ed., 2nd Edition, Acad. Press, Inc., San Diego, CA, USA, 1998)). The starting material, sample of plasma membranes, or disrupted plasma membrane sample, can also be subjected to a separation method based on a physical characteristic of the membranes, such as buoyant density, size, phase separation, to fractionate the sample. In a preferred method, the

25   sample can be subjected to a separation method based on density. Representative separation methods based on density include sucrose density gradient centrifugation, and centrifugation using an Opti-Prep gradient (Gibco, Inc. (Grand Island, New York). If a separation step is performed, it results in the production of fractions of components. Fractions which contain the greater amount of plasma membranes, with a lesser amount

of contaminants, are isolated for further use. For example, separation based on density results in the production of fractions having different densities. Of these fractions, the initial fractions are collected. "Initial fractions," as used herein, refer to the top (i.e., the least dense) several fractions, that constitute approximately 10-25% of the fractions. In

5 a preferred embodiment, the initial fractions constitute approximately 10-20% of the fractions. If the sample is fractionated based on another characteristic of the plasma membranes besides density, the appropriate fractions (i.e., the fractions that comprise the majority of the plasma membranes with minimal contamination from other components) are collected and used as the "initial fractions".

10 The starting material, sample of plasma membranes, disrupted plasma membrane sample, or the initial fractions are subjected to an immunoisolation method to isolate the purified caveolae. The terms, "immunoisolation" and "immunoaffinity isolation," used interchangeably herein, refer to separation of a component of interest (e.g., caveolae) from other materials using an antibody that is specific for (binds

15 preferentially to) the component of interest. In immunoisolation, a sample of interest (i.e., the starting material, sample of plasma membranes, disrupted plasma membrane sample, or the initial fractions) that comprises plasma membranes is incubated with antibody that is specific for the component of interest; the component of interest, which is bound to the antibody that is specific for the component of interest, is then separated

20 (isolated) from materials (components) that are not bound to the antibody. In a preferred embodiment of the invention, the incubation period of the immunoisolation method is a brief time, that is, the incubation is under about two hours, preferably about one hour or less. The brief time frame allows separation of purified caveolae that more closely resemble caveolae in their native state; as described in the Examples below.

25 Extended immunoisolation results in significant loss of various caveolar components, including loss of the proteins eNOS and heterotrimeric G proteins into solution, and increases adsorption of nonspecific proteins and/or contaminating membranes.

In the immunoisolation method, an antibody that is specific for caveolin is used. The antibody that is specific for caveolin must be an antibody that binds preferentially

to oligomeric caveolin, as caveolin is found in an oligomeric state surrounding intact caveolae (Scherer, P.E. *et al., J.Biol. Chem. 272(46)*:29337-46 (1997)). In a preferred embodiment, the antibody binds to caveolin in its native state, and also binds to caveolin in its monomeric state (e.g., in a monomeric state such as after denaturation during

5   Western blot analysis). The antibody allows separation of caveolae from other materials in the sample of interest. Caveolae that have been separated from other materials in the sample of interest are referred to herein as "purified caveolae". Purified caveolae can be bound to the antibody that is specific for caveolin, or can, if desired, be separated from the antibody.

10   Representative immunoisolation methods include isolation using the antibody specific for caveolin, where the antibody is coated on (attached) to a solid phase, such as beads or spheres. In a preferred embodiment, magnetic beads coated with the antibody are used. The materials that are bound to the antibody that is specific for caveolin (i.e., the purified caveolae), are separated from those materials in the sample of interest that

15   are not bound to the antibody. For example , if a solid phase (e.g., magnetic beads) coated with antibodies is used for immunoisolation, the solid phase (e.g., the beads) together with materials bound to the antibodies that are coated with the solid phase (i.e., the purified caveolae), are separated from materials that are not bound to the solid phase. The purified caveolae can be released from the solid phase, if desired, using

20   standard techniques.

The purified caveolae can be used for the identification of molecules and proteins which are involved in intra-, inter- or trans-cellular transport, in order to utilize such molecules and proteins for delivery of an agent (e.g., an antibody, a drug, a diagnostic agent or a gene) for treatment or therapy. Agents which target a particular

25   component of caveolae may be more easily delivered to the cell and, if desired, into the cell, across one side of a cell to the other side (e.g., across a an endothelial cell layer). Thus, these molecules and proteins can be used to transport agents into and across cell membranes, such as into and/or across the endothelium and, as a result, across the endothelial barrier. The purified caveolae can also be used to target the endothelium,

such as for delivery of an agent for treatment or therapy. Agents which target caveolae may also be more easily delivered to the cell and, if desired, into the cell, across one side of a cell to the other side (e.g., across a an endothelial cell layer). Thus, the caveolae can be used to transport agents into and across cell membranes, such as into

5 and/or across the endothelium and, as a result, across the endothelial barrier. This is of considerable value because of the role the endothelium plays in many tissues of the body as a barrier to the passage of substances across the endothelium and into the underlying tissue. For example, agents (e.g., antibodies, drugs, diagnostic agents, genes) which bind to the caveolae or to particular components (e.g., molecules or

10 proteins) can be used to target the caveolae. Alternatively, such agents can be used as transport agents, by conjugating another agent (e.g., a drug or a gene) to the agent which targets the caveolae.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings

15 of all references cited are hereby incorporated herein by reference.

EXAMPLE 1 Materials and Methods

Materials

Antibodies against caveolin (rabbit polyclonal and mouse monoclonal, clones #2297 and 2234, respectively) were purchased from Transduction Labs (Lexington,

20 KY), or from Zymed (South San Francisco, CA, mouse monoclonal #Z034). M-450 Dynabeads were purchased from Dynal (New Hyde Park, NY). All other reagents and supplies were obtained as described previously (Schnitzer, J.E. *et al., J. Biol. Chem.* *270*:14399-14404 (1995); Schnitzer, J.E. *et al., Science 269*:1435-1439 (1995); Schnitzer, J.E. *et al., Proc. Natl. Acad. Sci. USA 92*;1759-1764 (1995); Schnitzer, J.E. *et*

25 *al., Science 274*:239-242 (printer's erratum, *Science 274*:1069) (1996)).

Western and Protein Analysis

The proteins of various tissue fractions were solubilized and separated by SDS-PAGE for direct analysis by silver staining or for Western analysis by electrotransfer to nitrocellulose filters followed by immunoblotting using enhanced chemiluminescence

5   autoradiography and densitometric quantification using ImageQuant, as described previously (Schnitzer, J.E. *et al., J. Biol. Chem. 270*:14399-14404 (1995); Schnitzer, J.E. *et al., Science 269*:1435-1439 (1995); Schnitzer, J.E. *et al., Proc. Natl. Acad. Sci. USA 92*;1759-1764 (1995); Schnitzer, J.E. *et al., Science 274*:239-242 (printer's erratum, *Science 274*:1069) (1996)). Briefly, nitrocellulose filters from each gel were

10   probed using primary antibody (dilution range from 1:1,000 to 1:5,000 in Blotto) followed by the appropriate HRP-labeled reporter antibodies (diluted 1:1,000). Sample protein concentrations were measured using the micro-BCA method with BSA as a standard.

Purification of Slilca-Coated Endothelial Cell Plasma Membranes and

15   *Caveolae*

The luminal endothelial cell plasma membranes and caveolae were purified directly from rat lung tissue using an *in situ* silica-coating procedure as described previously ((Schnitzer, J.E. *et al., J. Biol. Chem. 270*:14399-14404 (1995); Schnitzer, J.E. *et al., Science 269*:1435-1439 (1995); Schnitzer, J.E. *et al., Proc. Natl. Acad. Sci.*

20   *USA 92*;1759-1764 (1995)). Rat lung tissue was subfractionated to isolate first the luminal endothelial cell plasma membranes, and then to dislodge and isolate caveolae (Schnitzer, J.E. *et al., J. Biol. Chem. 270*:14399-14404 (1995); Schnitzer, J.E. *et al., Science 269*:1435-1439 (1995); Schnitzer, J.E. *et al., Proc. Natl. Acad. Sci. USA 92*;1759-1764 (1995)). The rat lung microvasculature was perfused *in situ* at 10-13°C

25   via the pulmonary artery with a solution of positively charged colloidal silica particles which coated the intimal endothelial cell surface membrane directly in contact with the circulating blood and created a stable silica pellicle that specifically marked this membrane and facilitated its purification from tissue homogenates by centrifugation.

Electron microscopy showed that the sedimented pellets (P) contain silica-coated endothelial cell plasma membranes with many associated caveolae and little detectable contamination from other tissue components. Biochemically, P displayed ample enrichment for various endothelial cell surface markers relative to the starting whole

5   lung homogenate (H) while excluding various other proteins found in intracellular organelles or other cell surfaces. The caveolae attached on the cytoplasmic side of the membranes opposite to the silica coating were stripped from these membranes by shearing during homogenization at 4 °C. then, they were subjected to sucrose density centrifugation to yield a low buoyant density fraction of intact caveolar vesicles (V) well

10   separated from the pellet containing resedimented silica-coated membranes stripped of the caveolae (P-V) and enriched in caveolar but not other markers. Alternatively, caveolae were separated from the plasma membranes (P) by sonication and then isolated in a low buoyant density fraction (referred to herein as PC) by sucrose density gradient centrifugation.

15   *ELISA*

The reactivity of caveolin antibodies with intact caveolae on the purified, silica-coated endothelial cell plasma membranes was assessed by ELISA. Briefly, equal aliquots of P (silica-coated luminal endothelial cell plasma membranes, isolated as described above) (5 μg in 100 μl) were placed in each well of a 96-well tray for drying

20   overnight. After washing, the wells were blocked for 1 hour with EWB (2% ovalbumin and 2 mM $CaCl_2$ in PBS), incubated with EWB alone or EWB containing caveolin antibody, washed, incubated with reporter antibody conjugated to horseradish peroxidase (1:500 in EWB), and washed again. A substrate solution (50 mM Na_2HPO_4, 25 mM citric acid, 1.2 mg/ml of o-phenylenediamine dihydrochloride and 0.03% H_2O_2)

25   was added and the reaction was stopped with 4 M H_2SO_4 before reading the signal using a Molecular Device Thermomax microplate reader.

Immuno-affinity Isolation of Caveolae

Magnetic immunoisolations were performed as follows: M450 DynaBeads conjugated with anti-mouse or -rabbit IgG were washed 3 times with PBS by resuspension and magnetic separation and then incubated for 4-8 hours with the desired

5   antibody (10^7 M450 beads and 25 µg IgG). After washing 3 times with PBS, the beads were resuspended and incubated for one hour at 4°C with various starting membrane subfractions (SM) before washing and magnetic separation to isolate two fractions: material bound to the beads (BS) and material not bound to the beads (UB). The SM, BD and UB fractions were subjected to SDS-PAGE and Western analysis. All

10   experiments described herein were performed without added BSA as a blocker.

Simplified Purification of Caveolae from Plasma Membranes

Homogenates of tissue or cultured cells were subjected to Percoll gradient centrifugation to isolate the plasmalemmal fraction, in order to provide a sample enriched in plasma membranes. Lung tissue was homogenized in buffer (0.25 M

15   sucrose/1 mM EDTA/20 mM Tricine, pH 7.8) and the lung homogenate was filtered sequentially through 53 and 30 µm Nytex filters. The cell/filtered tissue homogenates were subjected to centrifugation at 4°C and the supernatant was saved. The resulting pellet was resuspended in 3 ml of buffer and subjected again to homogenization and centrifugation as above. The two supernatants were then combined and mixed with 30

20   ml of 30% Percoll in buffer. After centrifugation with a SW28 rotor at 84,000xg for 45 minutes at 4°C (no brakes), a single membranous band was collected. The band was readily visible about 2/3 from the bottom of the tube. The band was pelleted by diluting the suspension 2-3 fold with MBS before centrifugation at 15,000xg at 4°C for 2 hours. This band, referred to herein as the PM (plasma membrane) fraction, is enriched in

25   plasma membranes.

To isolate the caveolae, the PM fraction was resuspended in 1 ml Mes-buffered saline (MBS) and sonicated on "high" (2 x 10 seconds; Branson sonicator), followed by immediate cooling on ice. The sonication procedure was repeated 3 times. The

sonicated membranes were mixed with 50% Opti-Prep to yield a final concentration of 23% in a 3 ml total volume. After adding a continuous layer of 20-10% Opti-Prep to the SW 55 tube, the suspension was spun at 23,500xg for 90 minutes at 4°C in a SW 55 rotor. Depending on the amount of PM loaded, 5-7 distinct membranous bands were

5   visible. The first 2 bands (the "initial fraction"), or the top fourth of the tube, were then collected, and the membranes were pelleted by dilution and centrifugation as described above.

For immunoisolation of the caveolae, this membrane pellet was resuspended in 100 μl MBS for incubation with anti-mouse IgG Dynabeads (2 x 10^7 beads) prebound

10   with 25 μg of CAV and processed as described above.

EXAMPLE 2 Characterization of an Antibody Reactive with the Caveolin Cage of

Caveolae

Monoclonal antibody clone 2234(Transduction Labs, Lexington, KY), referred to herein as CAV, is not only specific for caveolin, but also binds caveolin in its native

15   state as an oligomeric structural cave surrounding intact caveolae. Previous work (Scherer, P.E. *et al., J. Biol. Chem. 270*:16395-16401 (1995)) indicated that this antibody specifically binds the α-isoform of caveolin-1 via a specific epitope found in the N-terminal segment that is not present in the β-isoform.

ELISA analysis, as described above, revealed that CAV was able to bind

20   caveolin expressed on the purified silica-coated endothelial cell membranes (P); in addition, kinetic and binding analysis indicated rapid and high affinity binding of CAV to the caveolae in P. Titration of antibody concentration demonstrated that binding was saturable, with maximum binding occurring at about 10 nm. Time course studies revealed that binding equilibrium was achieved rapidly in just 1 hour (Figure 1). Other

25   monoclonal antibodies (2297 and Z034), also specific for caveolin by Western analysis, showed very little to no binding to caveolin in P by ELISA (Figure 2). Polyclonal antibody showed a little reactivity, but only at higher concentrations and at levels insufficient to be useful for immunoisolation (Figure 2).

The ability of CAV to react with fraction V (the low buoyant density fraction containing caveolae, isolated from P as described above) was then examined by performing immuno-affinity isolation of the caveolae as described above. Results indicated that CAV bound to intact caveolae in V, to allow immunoisolation. Greater

5   than 95% of the caveolin in V was bound to the CAV-coated beads in the bound (BD) fraction, with little remaining behind in the unbound (UB) fraction. Furthermore, nearly all of the proteins detected in V fractionated to BD, rather than UB, upon CAV immunoisolation of the caveolae.

In contrast, other caveolin antibodies (2297, Z034) were ineffective, with nearly

10   all of the caveolin and protein signal detected in UB rather than BD. The polyclonal antibody was poor, but partially effective. CAV thus appeared to be a high affinity antibody reacting quite rapidly and specifically with caveolin not only in its monomeric state after denaturation during Western analysis, but also in its native oligomeric state surrounding intact caveolae found in P and V.

15   EXAMPLE 3 Comparison of Sonication and Shearing for Isolation of Low Density
Membranes

A comparison of the efficacy of sonication and of shearing in removing and isolating caveolae from the silica-coated cell plasma membranes (P) was performed, as described above. Sonication and shearing were both quite effective in not only

20   removing caveolae from P (>80% loss in caveolin signal in the resedimented silica-coated membranes), but also yielding a caveolin-enriched, low density vesicular fraction after continuous sucrose density centrifugation. Western blot analysis indicated that, relative to P, the V fraction (obtained by shearing) and the PC fraction (obtained by sonication) were both quite enriched in caveolin by more than 10-fold. Although PC

25   and V had many proteins in common (eNOS, G proteins, annexin II and PKC), many other proteins found in P were readily detected in PC but not V, including the cytoskeletal protein β-actin, the GPI-anchored protein urokinase-plasminogen activator receptor (uPAR) and angiotensin converting enzyme (ACE). Neither ϵ-COP (markedly

depleted in P) nor the GPI-anchored 5'-nucleotidase (5'NT) (enriched in P) were apparent in V or PC. Thus, V was quite distinct in molecular composition from P and PC.

SDS-PAGE gels further confirmed the differences between the fractions: V had
5   the simplest profile, with the fewest detectable proteins, whereas PC had many more protein bands than V but less than that seen in P. Although PC contained many, if not all, of the proteins apparent in V, it also contained many proteins found in P but not in V. This analysis indicated that sonication and shearing of P yielded vesicular fractions with the same low buoyant density but quite different molecular composition. It
10   appeared that sonication of P released additional low density vesicles not found in V.

EXAMPLE 4 Immunoisolation from PC and V with CAV Antibody

The PC and V fractions described above were subjected to identical immunoaffinity isolation, as described above, for one hour using CAV bound to magnetic beads. The starting material (referred to as "SM"; SM here was either PC or
15   V), the material not bound to the immuno-magnetic beads (UB) and the material bound to the beads (BD) were examined by Western blot analysis. The fractions were analyzed first to assess the relative distribution of the molecules by using equivalent volume conditions (the final volumes were equal in each fraction and the same volume of each fraction was added to the gel lanes, so that the same percentage or proportion of
20   each fraction was analyzed).

Although ample signals for each of the assessed molecules were seen in the starting material SM (PC), only a subset of these molecules were found in BD, namely, the vesicles bound to the caveolin-antibody beads. Caveolin gave the strongest signal in BD relative to both UB and SM. PKCα and the G protein $G_{\alpha q}$, as well as to a lesser
25   extent eNOS and $G_{\alpha s}$, were detected in BD, yet significant levels of each remained behind in UB. Little to no signal for annexin II, β-actin and uPAR was detected in BD; these proteins remained nearly completely in UB.

The results where fraction V was used as the starting material [SM (V)] differed significantly. In each case where a signal was present in SM (V), little to no signal was found in UB and nearly all of the signal was recovered in BD. For example, more than 95% of the signal in SM (V) for caveolin, G proteins, eNOS and PCKα was found in

5 BD. uPAR and β-actin were not detected in any of these fractions. Thus, nearly all of the caveolin was accessible and able to interact with the immuno-beads, resulting in quantitative isolation of nearly all of the starting material. This is in contrast with the results when PC was used as the starting material, where a significant amount of caveolin was inaccessible to immunoisolation.

10 To be certain that proteins in PC, for which a mild to nil signal was detected in BD, were not simply diluted out by maintaining equivalent volume conditions during the analysis, equal amounts of protein from each fraction were loaded onto the gels before Western analysis. This resulted in a signal for eNOS, G protein and annexin II that was more readily detected in BD from PC as the starting material. By contrast,

15 little to no β-actin or uPAR was again detected in BD, with ample signal present in UB, consistent with these molecules not normally residing concentrated in caveolae. Analysis of fractions under volume vs. protein equivalence gave noticeably different results, revealing readily apparent differences between PC and V, as well as striking similarities between V and the immunobound fractions (UB) from both PC and V.

20 EXAMPLE 5 Immunopurification of Caveolae from Plasma Membrane Isolated
without Silica Coating

Rat lungs flushed free of blood were processed to isolate a plasma membrane fraction (PM) using Percoll gradient centrifugation, and then a caveolin-enriched fraction (AC) was isolated by sonication of PM followed by sucrose density

25 centrifugation, as described by Smart et al. (Smart, E.J. et al., Proc. Natl. Acad. Sci. USA 92:10104-10108 (1995)). Western analysis was performed, revealing that PM was enriched in various plasmalemmal marker proteins including caveolin, 5'NT, β-actin, eNOS, and uPAR. PM also contained significant levels of ε-COP, indicating the

presence of contaminating Golgi and endosomal membranes. When PM was subfractionated as described by Smart *et al.* (supra) by sonication to produce small vesicles that were isolated by centrifugation, first as a broad band on a continuous sucrose gradient for separation, and then again on a step gradient effectively to

5   concentrate the low density buoyant vesicles, the resulting AC fraction was quite enriched in caveolin and eNOS (>5-fold) which is consistent with past reports (Shaul, P. *et al., J. Biol. Chem. 271*:6518-6522 (1996)). It was also mildly enriched in β-actin and the GPI-anchored proteins 5'NT and uPAR, again consistent with past studies (Smart *et al., supra*). Although not enriched, molecules such as annexin II as well as ε-COP were

10   easily detected in the AC fraction. The proteins detected in silver-stained gels for AC and PM were similar but clearly not identical.

AC was also subjected to immunoisolation using the CAV antibody, as described above. Western analysis of the starting material SM (AC), as well as the UB and BD fractions, under equal protein conditions indicated that many proteins originally

15   detected in AC did exist in the immunoisolated caveolin-coated vesicles (i.e., the caveolae), whereas many other proteins in starting material AC were not constituents of these vesicles. Caveolin in SM (AC) was found enriched in BD relative to UB. eNOS was clearly detected in BD with little signal in UB. Annexin II partitioned approximately equally between SM (AC), UB and BD. The GPI-anchored proteins

20   5'NT and uPAR, as well as the Golgi/endosomal marker ε-COP and the cytoskeletal protein β-actin were not readily detected in BD, but rather, remained in UB. To be certain that the GPI-anchored proteins did not dissociate from membranes into solution, the UB fraction was subjected to centrifugation. It was found that the GPI-anchored proteins were in the membrane pellets. Thus, CAV immunoisolation purified the

25   caveolin-coated caveolae in the AC fraction away from other membrane microdomains, including those rich in GPI-anchored proteins and/or cytoskeletal proteins.

EXAMPLE 6 CAV Immunoisolates Nearly All Proteins and Caveolae in V, but not PC
or AC

The low density, caveolin-rich vesicles isolated from each fractionation
procedure (V, PC and AC), were subjected identically to immunoisolation for one hour
5 using CAV before performing SDS-PAGE as well as Western analysis for caveolin on
the immuno-separated fractions. The gels revealed that nearly all of the membranes in
V bound to the CAV beads, and that nearly all of the proteins as well as caveolin in V
fractionated with the immuno-beads in BD, with little to none remaining in the UB
fractions. Except for some bands coming from the IgG heavy and light chains, and BSA
10 included by the manufacturer with the beads, the protein profile of V in SM was
identical to BD. Conversely, when CAV was replaced with a clathrin antibody as a
control, little to none of the proteins and caveolin originally in SM (V) was detected in
BD, but remained in UB.

For PC and AC, the results were strikingly different. Proteins were readily
15 detected in both the BD and UB fractions, consistent with the presence of a significant
population of low density vesicles that were not reactive with the caveolin antibody.
Increasing the amount of antibody and immuno-beads several fold yielded the same
result. The protein profiles in BD and UB were quite different, yet the proteins detected
in BD from PC and AC were very similar to each other, as well as to those seen in V
20 (also BD from V). Thus, subjecting the AC and PC fractions to immunoisolation
revealed the existence of a heterogeneous population of distinct vesicles rather than a
homogeneous population of caveolin-coated vesicles.

This analysis was in concordance with the Western analysis (above), that
showed that many molecules in SM (PC) or SM (AC) were split between UB and BD,
25 whereas for V, all molecules in SM (V) fractionated to BD with little to none remaining
in UB. The same proteins detected in V (before immunoisolation as well as after) were
found in the immunoisolated caveolae from PC and AC. These results indicated that V,
but not AC nor PC, contained a homogeneous population of purified caveolin-coated
caveolae. For PC and for AC, immunoisolation was a necessary additional step to

purify caveolae. The caveolae immuno-purified from PC and AC appeared to be biochemically identical to each other as well as to those of V.

EXAMPLE 7 Time-dependent Translocation of eNOS into Solution

Overnight immuno-isolations, and 1 hour immunoisolations, were performed as

5   described above in Example 6, to assess the ability of CAV antibody to isolate caveolae. Immunoblots of immunoseparated fractions indicated that although caveolin was almost completely detected in BD for both the 1 hour and overnight incubations, eNOS and G_{as} were in BD in the 1 hour incubation, but largely absent from BD after the overnight incubation. Although it was clear that eNOS and G_{as} were initially a part of the floating

10   low density vesicles in V, the overnight incubation caused a substantial release of these signaling molecules from the caveolin-coated caveolae into solution. This apparent dissociation was confirmed in experiments performed in the absence of antibody, where equal aliquots of P were placed in solution for times ranging from minutes to 16 hours, before sedimenting the membranes at 100,000xg and processing the nonparticulate

15   supernatants and membrane pellets for Western analysis. As early as 2 to 4 hours, a loss in eNOS and G_{as}, but not caveolin, was detected from the pelleted membranes, with a concomitant gain in the supernatant. At 1 hour or less, all of the signal detected for these three proteins was detected in the membrane pellet. Thus, with time, these lipid-anchored signaling molecules dissociated from the membrane into solution.

20   EXAMPLE 8 Simplified Immunopurification of Caveolae From Plasma Membranes

A simplified approach to purifying caveolae from cells and tissues was developed. First, a plasma membrane-enriched fraction, equivalent to PM described above, was isolated from tissue and/or cell homogenates by standard centrifugation techniques (e.g., using Percoll gradients). This PM was subjected to vigorous

25   sonication, and then loaded onto a continuous Opti-Prep gradient for centrifugation at 52,000xg for 90 min. The top 25% of the gradient (first 4 fractions) was collected. This caveolin-rich fraction, which contained much less ε-COP and GPI-anchored proteins

than vesicles floating at higher densities, was then subjected to immunoisolation using CAV antibody.

This procedure yielded purified caveolin-coated caveolae equivalent in molecular composition to those derived by CAV immunoisolation of PC, V, and AC.

5 This procedure shortened the procedure by avoiding the additional Opti-Prep centrifugation step as performed in the AC isolation.

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the

10 scope of the claims.